REMARKS

I. Description of the Present Invention

The present invention relates to a method for detecting a target nucleic acid sequence. The method of the present invention may employ both an amplification procedure and a detection procedure. A plurality of pairs of nucleic acid amplification probes is used to effect amplification of the target sequence. The member probes of each pair of amplification probes are complementary to each other with at least one same hybridizing member of each pair of amplification probes also being complementary to a portion of the target nucleic acid sequence, which acts as a template. The hybridizing members of each pair of amplification probes hybridize to the target sequence in a contiguous manner, such that the amplification probes can be ligated to form an amplification product which is made up of a plurality of amplification probe segments. Following ligation of the hybridized amplification probes, the completed amplification product is separated from the template (i.e., denatured). The process is then repeated, with amplification product also acting as a template in subsequent cycles, until a sufficient quantity of the target nucleic acid sequence is produced to result in measurable signal in the selected assay.

Where three or more pairs of amplification probes are used in the amplification procedure, the resulting amplification product may be specifically detected using two or more detection probes of the present invention. Each detection probe is complementary to a portion of each of a different combination of two adjacently situated amplification probe segments of the amplification product. The correctly assembled amplification product thus serves as a template for the detection probes in a manner similar to that served by the target nucleic acid sequence in the amplification procedure. The detection probes hybridize

to the amplification product in a contiguous manner; i.e., sufficiently adjacent to each other to enable the hybridized detection probes to interact with each other to ultimately produce a detectable signal. As opposed to the amplification probes, which require ligation for the formation of amplification product, the detection probes need not be ligated in order to form detection product.

The detection system of the present invention enables one to discriminate between the correctly assembled template-derived amplification product and the incorrectly assembled blunt-end ligated spurious amplification by-product inherently generated from the amplification system, thus dramatically improving the overall sensitivity of the method. By minimizing the deleterious effect caused by the concurrent production of increasing amounts of spurious amplification by-product, the full benefit of improved sensitivity from increased numbers of pairs of amplification probes can be realized.

II. 35 USC §112 Objections

Amendment of the claims is made herein to address the Examiner's objections under 35 USC §112.

A. 35 USC §112, Second Paragraph

The Examiner has objected to claims 1, 14, 19, and 21 on the grounds that these claims, as originally drafted, allegedly allow for the possibility that the plurality of pairs of amplification probes can be in a hybridized form, "...causing an inoperability condition to arise in subsequent steps since hybridization cannot occur with already hybridized member probes." Claims 1 and 14 have been amended to reflect that a plurality of denatured pairs of amplification probes participates in the amplification of the target sequence. Inclusion of the adjective "denatured" to modify the pairs of amplification probes

reflects the teachings of the specification and addresses the Examiner's objections to claims 1 and 14.

Pairs of amplification probes can, however, be added to the reaction mixture of the amplification system of the present invention in hybridized form. In such an instance, the hybridized pairs of probes would be denatured subsequent to their addition to the reaction mixture in order to enable the amplification probes to hybridize to the target sequence. In point of fact, it may actually be advantageous to add the pairs of amplification probes in hybridized form, in order to ensure that the pairs of probes are "paired", as taught in the present specification.[1] For this reason, reagent claims 19 and 21 have not been amended to include the limitation that the plurality of pairs of amplification probes be provided in denatured form. In the case of these reagents, it is unnecessary that the pairs of probes be denatured in advance of being added to a reaction mixture, inasmuch as a denaturing step can easily be added to the amplification procedure.

The Examiner has also objected to claims 1, 14, 19, and 21, because the ratio of amplification probe to amplification sequence "...is unstated so self-annealing of the amplification probes can occur with method-defeating regularity". Claims 1, 14, 19, and 21 have therefore been amended to expressly state that an excess of a plurality of pairs of amplification probes sufficient to drive the reaction forward are provided. The specification states at page 15, line 33, that the amplification probes are "provided in excess for amplification of the

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[1]Where the amplification probes are not paired (e.g., not in molar balance; i.e., 1:1 ratio), the member probe which is provided in excess can compete with the template sequence for hybridization with the corresponding member probe which is provided in lesser quantity, thus decreasing the efficiency of the amplification system.

amplification sequence". While the examples teach the use of amplification probe in excesses over amplification sequence covering a range of from about 150:1 to 2,000:1 in the particular assay configurations demonstrated, the exact relative concentration ranges employed in a particular assay to provide optimal results will be apparent to those of ordinary skill in the art. It should be noted, however, that there will always be some degree of self-annealing where pairs of amplification probes are provided in excess of the target.

The Examiner has also objected to claims 1 and 14 on the grounds that these claims, as originally drafted, allow for the possibility that at least some of the plurality of pairs of amplification probes can be identical, noting that "...the outcome of such an occurrence is unclear." The Examiner is correct in noting that some of the pairs of amplification probes may be identical, but this will occur only in the rare instance where there are tandem repeats in a target sequence, as is known to occur in some viruses. In order to avoid confusion, and possible inconsistency within the claim language, the limitation that the amplification probes hybridize to a "different" portion of the amplification sequence has been eliminated from claims 1, 14, 19, and 21. It is important to remember, as taught in the specification, that the amplification system of the present invention operates on the basis of kinetics and complementarity of the amplification probes to the amplification sequence. If, for example, the second and third pairs of probes are selected to be identical, as might be the case in the unusual situation of tandem repeats, then twice as much of this particular pair of probes is added to the reaction mixture. The outcome is essentially the same as with any other assay configuration for carrying out the amplification system of the present invention.

The Examiner has objected to claims 1 and 14, step (b), because it is allegedly unclear whether the amplification probes can be incompletely hybridized to the amplification sequence.

According to the Examiner, incomplete hybridization of the probes to the amplification sequence can result in hybridization of the probes with each other. In the first place, the excess of the plurality of pairs of amplification probes provided in accordance with the present invention drives the reaction forward and essentially eliminates this possibility. As previously noted, the claims have been amended to reflect that an excess of pairs of amplification probes is used in the amplification system of the present invention. Secondly, step (b) specifically recites "...allowing said hybridizing member of said amplification probes to hybridize to..." the amplification sequence. [Emphasis added.] It is not understood how this claim language is construed by the Examiner to encompass partial hybridization.

The Examiner further states, with respect to step (e) of claims 1 and 14, that "...the result will be only a number of complementary copies of the amplification sequence if this sequence is a single strand originally, since there is no provision for hybridization to a complementary sequence in the preceding steps." If the amplification sequence is single stranded originally, there can be no hybridization to a complementary sequence in the preceding steps, because there is, in fact, no complementary sequence to which the non-hybridizing members of the pairs of amplification probes can hybridize. Hence the designation of "hybridizing members" of pairs of amplification probes in the specification. In such an instance, just as in the case of a double stranded target sequence, the complementary copies of amplification product from the first cycle act as a template in subsequent cycles to generate amplification product. This is more fully explained in the specification at page 14, line 16 to line 34. For further clarification and consistency, claims 1, 14, 19, and 21 have been amended to recite that the amplification sequence acts as template sequence, and that the pairs of amplification probes hybridize to template sequence in subsequent cycles.

The Examiner has also objected to claims 1, 14, 19, and 21 on the basis that the meaning of "same" in "same hybridizing member" is allegedly unclear. This term is, however, clearly defined in the present specification at page 8, line 11 to line 17, wherein it is stated that "same member" is that member of each pair of probes which is cumulatively capable, with other "same members", of forming a complete amplification product. In other words, with respect to a plurality of pairs of amplification probes, the "same members" will be either the member of each pair of probes which originates from the "upper strand", or the member of each pair of probes which originates from the "lower strand".

Step (c) of claim 14 has been amended to specify that the amplification product formed from the ligation of hybridized amplification probes is composed of amplification probe segments, thus providing antecedent basis for the reference to "amplification probe segment" in step (e).

With respect to claim 14, the Examiner has also alleged that it is unclear how more than one amplification probe member can hybridize to an amplification sequence of step (a). Where the amplification sequence is single stranded, only one member of each pair of probes will hybridize to the amplification sequence. Both members of the pair will, however, hybridize to template sequence during subsequent cycles of amplification. In the case of double stranded amplification sequence, both members of the pair can hybridize to target sequence during the first cycle.

Claims 6, 14, and 21 have also been objected to as allegedly vague and confusing. Specifically, it is alleged that in step (b), it is unclear what constitutes "an interaction". Although it is believed that the meaning of this term is described in the specification, claims 6, 14, and 21 have been amended to delete reference to binding of the detection probes

sufficiently adjacent to each other to enable "an interaction" to occur. Instead, reference to "contiguous" binding of the detection probes to form a "detection product" has been substituted therefore. These terms (i.e., "contiguous" and "detection product") are defined in the specification and characterize the nature of the binding of the detection probes of the present invention.

Step (c) of claims 6, 14, and 21 has also been objected to, because there is no positive recitation of the detection procedure. Claims 6, 14, and 21 have now been amended to indicate that at least one of the detection probes is labeled. The detection procedure is now positively recited as measuring the presence of detection product through the presence of the label.

Finally, claims 6, 14, and 19 have been amended to recite that each of the two detection probes is complementary to a portion of a <u>different combination</u> of each of two of said ligated amplification probe segments which are adjacently situated in said amplification product. Characterization of the complementarity of the detection probes in this manner necessarily requires hybridization of the detection probes to different locations along the amplification product. It has further been clarified that at least two detection probes are used in accordance with the detection system of the present invention. While the Examiner states that it is unclear how the method allows detection of <u>three</u> or more ligated nucleic acid segments [emphasis the Examiner's], the detection of an amplification product having three amplification probe segments, in accordance with the teachings of the present invention, is illustrated in Figure 2, and demonstrated in Example 6.

The Examiner has objected to claims 7 and 8 as allegedly vague and confusing "...since it is unclear that the method distinguishes hybridized from nonhybridized labeled

probes...". Claim 7 has been amended to include the additional step wherein unhybridized labeled probes are separated from detection product. The previous limitation requiring that at least one of the probes be labeled is now omitted due to incorporation of this limitation into rewritten claim 6. It is unnecessary in the case of certain labeling systems, such as proximity labels as specifically recited in claim 8, that the unhybridized labeled probes be separated from the amplification product, because, in these instances, the unhybridized probes will not contribute to measurable signal. (I.e., the amplification product is required as a template to bring together the proximity-labeled detection probes to produce signal.)

Claim 9, by virtue of its dependence from claim 6 as rewritten, now gives a positive recitation of the detection method for the ligated detection product.

The Examiner states that claim 8 is further vague, because the meaning of "proximity" is unclear. This term is, however, clearly defined in the specification at page 10, line 26, and page 19, line 21 to page 20, line 10. The Examiner uses the term proximity labels in the Office Action at page 9, lines 13 to 16, wherein it is stated, "It would have been obvious to use proximity labels in the Whiteley-Palva procedure for the expected signal generated when the reactive components combine in the hybridization process."

A. 35 USC §112, First Paragraph

The Examiner has further rejected claims 1 and 14 under 35 USC §112, first paragraph, on the basis that the disclosure is enabling "only for claims limited to the joining of amplification probes by bonds that are not separated during subsequent steps." Claims 1 and 14 have been amended to recite that the hybridized amplification probes are ligated to form amplification product, as taught in the specification.

III. 35 USC §103 Objections

The Examiner has rejected all of claims 1-12 of the present application under 35 USC §103 on the basis of three references. A summary of the three prior art references cited by the Examiner is provided in advance of a discussion of the specific 35 USC §103 rejections.

A. Prior Art Cited by the Examiner

1. Whiteley et al

Whiteley et al disclose lengthening a labeled probe used for the detection of an immobilized target sequence. The Whiteley et al method is designed to obviate a background problem presented by either partial hybridization of the labeled detection probe to non-target nucleic acid or nonspecific binding, wherein the labeled detection probe sticks to the surface of the membrane or other material used for immobilization of the sample.

Specifically, the Whiteley et al method provides a lengthened detection probe which can be distinguished by automated methods from shorter spuriously bound detection probes. The lengthened probe is formed from the ligation of a labeled detection probe to a "contiguous" probe. The contiguous probe is selected to be complementary to a portion of the target sequence which is contiguous with the "diagnostic" portion to which the labeled detection probe hybridizes. More specifically, the Whiteley et al method entails: (1) contacting the immobilized target sample with both a labeled detection probe and a contiguous probe; followed by, (2) ligation of the detection probe to the contiguous probe; then, (3) raising the temperature of the reaction mixture to melt off the shorter spuriously bound detection probe; and finally, (4) further raising the temperature

to release the specifically bound lengthened probe formed from ligation of the labeled detection probe and contiguous probe. The Whiteley et al method takes advantage of the fact that the lengthened detection probe will not be susceptible to melting at the initial lower raised temperature, while the shorter, spuriously bound detection probe, will be removed from the reaction mixture, thus eliminating background caused by the presence of spuriously bound product. Furthermore, the Whiteley et al method is directed to solving the unique problems of an automated probe hybridization assay.

2. Mullis et al

Mullis et al disclose an amplification procedure known as PCR amplification wherein two oligonucleotide primers are used as reagents. The primers are designed to flank the DNA segment to be amplified. As discussed in the background of the present specification, the primers anneal to their complementary sequences, on opposite strands of the target. Extension products of the annealed primers are then formed in the presence of DNA polymerase. Because the primers bind to positions on the target sequence which flank the segment to be amplified, DNA synthesis by the polymerase proceeds across and through the region between the primers, effectively doubling the amount of the target DNA segment flanked by the primers. The extension products are then removed from the target by heat denaturation

Cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase are repeated, resulting in an exponential accumulation of the target nucleic acid sequence in the form of extension product.

3. Palva et al

Palva et al disclose the use of an alternating series of multiple capture probes with an alternating series of multiple

detection probes to remove and detect, respectively, target sequence from a test sample. The capture probes are selected to be near, but not adjacent, to one another with respect to the target sequence, so that they hybridize to noncontiguous portions of the target. This enables the interspersed unhybridized portions of the target sequence to extend out from the surface or medium on which the capture probes are immobilized, and thus be accessible to the alternating series of labeled detection probes. While the multiple capture probes may be ligated together, the ligation is for convenience only and is effected prior to immobilization of the capture probes on the solid support. The detection probes are not ligated to themselves or to the capture probes.

B. Nonobviousness of the Present Invention

The present invention is unobvious in light of the prior art. The 35 USC §103 objections raised by the Examiner represent a hindsight reconstruction of the invention using bits and pieces of information gleaned from the references cited by the Examiner. There is absolutely nothing in the prior art to suggest, in any way, combining the teachings of Whiteley et al, Mullis et al, and/or Palva et al. Even if there were such a suggestion, the mere combination of elements would not result in a working amplification system.

The Examiner has rejected claims 19-21 under 35 USC §103 as being unpatentable over Whiteley et al, although there is nothing in the Whiteley et al reference that so much as hints of the use of contiguous probes for the purpose of amplification. The Examiner alleges, however, that it "would have been obvious to provide the probes of Whiteley et al as pairs of complementary nucleic acid sequences because the probes were synthesized as the complementary strands of the target sequence, so it would have been a routine procedure to synthesize complementary strands of the originally synthesized probes." This observation fails to

take into account the unobvious premise of the present invention of using pairs of probes to achieve exponential amplification of a target sequence. While the synthesis of complementary strands of the "originally synthesized probes" may be a "routine procedure", the use of these complementary strands, as pairs of probes, to achieve amplification of a target is not.

In fact, converting the contiguous probes used in Whiteley et al into the form of pairs of probes would be an unobvious step, for whatever diagnostic purpose. Introduction of pairs of probes would result in the complementary member competing with target for hybridization to the corresponding member of the probe pair, thus decreasing the signal and overall sensitivity of the method.

The Examiner has also rejected claims 1-5 of the present application under 35 USC §103 as being unpatentable over Whiteley et al in view of Mullis et al. The Examiner states that it "would have been obvious to perform the procedure of Whiteley et al through several cycles in order to amplify the target sequence present in a sample in view of Mullis et al." The Examiner appears to cite the Mullis et al reference for the proposition that an amplification scheme employing cycling is obvious as long as the individual elements (e.g., probes, ligation) can be found in one or more other prior art references. Such an argument, however, represents a sweeping generalization that unfairly minimizes Applicants' contribution to the art. The Examiner goes on to state that it "would have been further obvious to include more probes in the Whiteley-Mullis procedure for the lower probability of unspecific probe hybridization as Whiteley et al disclose."

Mullis et al disclose only one method for amplifying a target sequence. There is nothing in the Mullis et al reference to suggest other methods for achieving amplification. The Whiteley et al reference, on the other hand, is simply an example

of the ligation of two contiguous probes in order to discriminate correctly bound lengthened detection probe (ligated product) from spuriously bound shorter detection probe. There is no suggestion, and certainly no teaching, in either Mullis et al or Whiteley et al, that the Whiteley et al procedure can be adapted in any way to achieve amplification of a target sequence. At best, Mullis et al disclose the use of primers and repeated cycling. There is no suggestion in either reference as to how the probes of Whiteley et al should be selected and aligned with respect to the target sequence in order to achieve amplification. If one were to perform the procedure of Whiteley et al through several cycles in order to amplify the target sequence in a test sample, as suggested by the Examiner, one would achieve linear amplification at best. Moreover, this linear amplification would be of the complement to the target, and not of the target itself.

The Examiner's suggestion that including more probes in the Whiteley-Mullis procedure would expectedly result in a lower probability of unspecific probe hybridization is likewise incorrect. Increasing the number of probes in Whiteley et al would result only in decreased sensitivity (by adding the requirement for an additional ligation event), and would not affect specificity at all. In point of fact, increasing the number of pairs of amplification probes in the amplification procedure of the present invention results in increased production of spurious blunt-end amplification by-product. Only the percentage of correctly assembled by-product decreases with increasing numbers of pairs of amplification probes. Applicants' ability to discriminate between correctly assembled by-product and incorrectly assembled by-product, through the detection procedure of the present invention, is anything but expected or obvious. It is this unexpected discriminatory capacity of Applicants' invention which enables Applicants to take advantage of increasing the number of pairs of amplification probes to achieve greater sensitivity.

Nowhere is this more evident than in European Patent Application No. 320,308 ("Backman et al"),[2] which discloses an amplification system, wherein multiple probes are ligated to form "reorganized fused probes", or amplification product. Backman et al teach that the use of four probes is "sufficient and preferred" in achieving amplification product through ligation and cycling. This preference for four probes apparently arises because of the limited solutions identified by Backman et al for preventing the formation of spurious blunt-end ligated amplification by-product. These solutions include: (1) phosphorylating only the abutting ends of the probes; and, (2) using probes of unequal length. These two solutions appear to be addressed to a four probe system, inasmuch as only the terminal probes are available for this type of manipulation. The only other solution presented by Backman et al for alleviating the blunt-end ligation problem is limiting the number of amplification cycles. This, in turn, limits the sensitivity of the amplification procedure, yielding a result which is self-defeating.

The present invention is not limited to two pairs of probes, but effectively makes use of increasing numbers of pairs of probes (three or more) to improve sensitivity, because of its ability to overcome, rather than merely avoid, the blunt-end problem. Applicants' amplification system thus provides an almost unlimited number of parameters available for adjustment, so that the amplification system of the present invention can be tailored to any one of a number of diagnostic situations. The ability to overcome the blunt-end problem enables much greater sensitivity to be achieved than is possible in a system which

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[2]The Backman et al reference, cited as of interest in the information disclosure statement filed herewith, claims priority from U.S. Patent Application Serial No. 131,936, filed December 11, 1987.

must rely on reducing the formation of blunt-end spurious by-product. This is particularly noteworthy in situations where the target nucleic acid is present in very minute quantities, which rises to the level of critical importance in the early detection of certain deadly viruses such as the HIV virus.

The Examiner has rejected claims 6-13, also under 35 USC §103, as being unpatentable over Whiteley et al in view of Palva et al. The Examiner asserts that the multiple capture probes of Palva et al, which may be ligated together, "can be constructed so that they will contiguously hybridize to the target sequence." This is in direct contradiction to the teachings of Palva et al. The Palva et al invention makes use of "at least two series" of alternating probes which are situated "close to but not adjacent to one another." The multiple capture probes comprise one "series". The detection probes comprise the other "series". The member probes of these two series are specifically designed to alternate in position with respect to the target sequence. While the multiple capture probes can be ligated together, for convenience in attaching the capture probes to an immobilizing support, they are expressly designed not to be contiguous. By design, the multiple capture probes of Palva et al are constructed to contiguously hybridize to the target sequence.

The Examiner's assertion that it "would have been obvious to use the technique of Whiteley et al to detect ligated nucleic acid sequences in view of Palva et al", is inapplicable to the present invention. In the first place, it is unobvious as to why one would choose to detect a series of noncontiguous ligated multiple capture probes. It is even more attenuated to assume that the mere detection of a series of ligated multiple capture probes would suggest the use of contiguous detection probes to identify correctly assembled amplification product. Further, there is no teaching as to how to use contiguous detection probes to detect correctly assembled amplification

product. By contrast, the present specification teaches how to select the detection probes to span amplification probe segment junctions of amplification product in order to discriminate correctly assembled amplification product from incorrectly assembled by-product. This enables Applicants to achieve any desired degree of sensitivity in an amplification/detection system simply by increasing the number of pairs of amplification probes. Applicants' ability to attain improved sensitivity by employing a practice (i.e., increasing the number of pairs of amplification probes) which operates against the teachings of the art, is perhaps the most persuasive argument for unobviousness.

The Examiner further states that claims 14-18 are rejected under 35 USC §103 as being unpatentable over Whiteley et al in view of both Mullis et al and Palva et al. In addition to the arguments previously made, there is no suggestion whatsoever of the use of ligation of multiple capture probes, as taught by Palva et al, to amplify target sequence.

For the foregoing reasons, Applicants believe the present application is in condition for allowance, and an early notification to that effect is earnestly solicited.

Respectfully submitted,

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